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(54) Title: ERYTHROPOIETIN ANALOG COMPOSITIONS AND METHODS

(57) Abstract

Analogs of human erythropoietin, including the [X^{33},Cys^{139},des-Arg^{166}] and [Cys^{139},des-Arg^{166}] analogs, as well as methods for making and using such analogs and pharmaceutical compositions containing the same.

ERYTHROPOIETIN ANALOG COMPOSITIONS AND METHODS

This application is a continuation-in-part of copending United States Patent Application Serial No. 08/055,076, filed on April 29, 1993.

Technical Field

The present invention relates to analogs of human erythropoietin, a glycoprotein known to be useful in inducing erythropoiesis and in treating conditions, such as anemia, which are due to low erythrocyte or reticulocyte count. The invention also relates to methods and compositions for making the analogs and methods of using the analogs to induce erythropoiesis and treat conditions, such as anemia, which result from inadequate erythrocyte or reticulocyte count.

Background Of The Invention

Erythropoietin is a naturally-occurring glycoprotein hormone with a molecular weight that was first reported to be approximately 39,000 daltons (T. Miyaki *et al.*, *J. Biol. Chem.* 252:5558-5564 (1977)). The mature hormone is 166 amino acids long and the "prepro" form of the hormone, with its leader peptide, is 193 amino acids long (F. Lin, U.S. Patent No. 4,703,008). The mature hormone has a molecular weight, calculated from its amino acid sequence, of 18,399 daltons (K. Jacobs *et al.*, *Nature* 313:806-810 (1985); J.K. Browne *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* 51:693-702 (1986)).

Structural characterization of human urinary erythropoietin has identified a des-Arg166 form that results from specific removal of the Arg residue at the carboxy-terminus of the mature protein (M.A. Recny *et al.*, *J. Biol. Chem.* 262:17156-17163 (1987)). Recny *et al.*, *supra*, propose that the physiologically active form of erythropoietin circulating in human plasma is the des-Arg166 form.

Human erythropoietin contains three N-linked carbohydrate chains (H. Sasaki *et al.*, *J. Biol. Chem.* 262:12059-12076 (1987); E. Tsuda *et al.*, *Biochemistry* 27:5646-5654 (1988); and M. Takeuchi*et al.*, *J. Biol. Chem.* 263:3657-3663 (1988)). The carbohydrate content of erythropoietin is similar in both naturally-occurring urinary erythropoietin and in hormone produced by expression, in mammalian cells in culture, of a cloned DNA which has been transfected into the cells and which encodes the prepro form of the hormone. The N-linked glycosylation sites are located at amino acid residues 24, 38, and 83. Both urinary and recombinant erythropoietin also contain a single O-linked glycosylation site at amino acid residue 126 (H. Sasaki *et al.*, *supra*; E. Tsuda *et al.*, *supra*; M. Takeuchi *et al.*, *supra* ; and M. Goto *et al.*, *Biotechnology* 6:67-71 (1988)). The carbohydrate content of erythropoietin is a

such as IL-3, granulocyte macrophage colony-stimulating factor and interleukin-9 are known to have burst-forming activity. However, it is unclear whether these activities have any physiological role in erythropoiesis (J. Suda *et al.*, *Blood* 67:1002-1006 (1986); C.A. Sieff *et al.*, *Science* 230:1171-1173 (1985); and R.E. Donahoe *et al.*, *Blood* 75:2271-2275 (1989). Recently, a factor termed "erythroid differentiation factor" has been shown to potentiate the activity of erythropoietin *in vivo* and *in vitro* (H.E. Broxmeyer *et al.*, *Proc. Natl. Acad. Sci.* 85:9052-9056 (1988); J. Yu *et al.*, *Nature* 330:765-767 (1987)). This factor has been shown to be identical to activin A (follicle-stimulating hormone-releasing protein) and to be inhibited by follistatin, a specific inhibitor of activin A; however, the physiological role of activin A remains to be determined (M. Shiozaki *et al.*, *Proc. Natl. Acad. Sci.* 89:1553-1556 (1992)). Thus, after nearly twenty years of investigation, there is no clear indication that erythropoiesis is controlled by any hormone other than erythropoietin.

In the absence of any alternative hormones which affect erythropoiesis, several attempts to both probe erythropoietin structure and significantly improve the characteristics of erythropoietin by site-directed mutagenesis have appeared in the literature. The molecular cloning of the human gene encoding erythropoietin reveals a DNA sequence coding for a preprohormone of 193 amino acids and a mature hormone of 166 amino acids. The availability of cloned DNA encoding the hormone and its precursor (*i.e.*, the prepro form) provides the opportunity for mutagenesis by standard methods in molecular biology. *See* U.S. Patent No. 4,703,008, *supra*.

The first mutant erythropoietins (i.e., erythropoietin analogs), prepared by making amino acid substitutions and deletions, have demonstrated reduced or unimproved activity. As described in U.S. Patent No. 4,703,008, replacement of the tyrosine residues at positions 15, 49 and 145 with phenylalanine residues, replacement of the cysteine residue at position 7 with an histidine, substitution of the proline at position 2 with an asparagine, deletion of residues 2-6, deletion of residues 163-166, and deletion of residues 27-55 does not result in an apparent increase in biological activity. The Cys^7-to-His^7 mutation eliminates biological activity. A series of mutant erythropoietins with a single amino acid substitution at asparagine residues 24, 38 or 83 show severely reduced activity (substitution at position 24) or exhibit rapid intracellular degradation and apparent lack of secretion (substitution at residue 38 or 183). Elimination of the O-linked glycosylation site at Serine126 results in rapid degradation or lack of secretion of the erythropoietin analog (S. Dube *et al.*, *J. Biol. Chem.* 33:17516-17521 (1988)). These authors conclude that glycosylation sites at residues 38, 83 and 126 are required for proper secretion and that glycosylation sites located at residues 24 and 38 may be involved in the biological activity of mature erythropoietin.

none show a significant increase in their ability to raise hemoglobin, hematocrit or reticulocyte (the immediate precursor of an erythrocyte) levels when compared to native erythropoietin.

Another set of mutants has been constructed to probe the function of residues 99-119 (domain 1) and residues 111-129 (domain 2) (Y. Chern *et al.*, *Eur. J. Biochem.* 202:225-230 (1991)). The domain 1 mutants are rapidly degraded and inactive in an *in vitro* bioassay while the domain 2 mutants, at best, retain *in vitro* activity. These mutants also show no enhanced *in vivo* biological activity as compared to wild-type, human erythropoietin. These authors conclude that residues 99-119 play a critical role in the structure of erythropoietin.

The human erythropoietin molecule contains two disulfide bridges, one linking the cysteine residues at positions 7 and 161, and a second connecting cysteines at positions 29 and 33 (P.-H. Lai *et al.*, *J. Biol. Chem.* 261:3116-3121 (1986)). Oligonucleotide-directed mutagenesis has been used to probe the function of the disulfide bridge linking cysteines 29 and 33 in human erythropoietin. The cysteine at position 33 has been converted to a proline residue, which, mimics the structure of murine erythropoietin at this residue. The resulting mutant has greatly reduced *in vitro* activity. The loss of activity is so severe that the authors conclude that the disulfide bridge between residues 29 and 33 is essential for erythropoietin function (F.-K. Lin, Molecular and Cellular Aspects of Erythropoietin and Erythropoiesis, pp. 23-36, ed. I.N. Rich, Springer-Verlag, Berlin (1987)).

Site-specific oligonucleotide-directed mutagenesis of the methionine residue at position 54 of human erythropoietin results in a molecule which retains the *in vivo* biological activity of the parent (wild-type) molecule with the added advantage of providing an erythropoietin preparation which is less susceptible to oxidation (Shoemaker, U.S. Patent No. 4,835,260).

A large number of mutants of the human erythropoietin gene have been described in several scientific publications and patent applications. These mutants have spanned the entire length of the molecule, have produced partially- or completely-deglycosylated molecules, have altered the structures of the disulfide bridges in the molecule, and have attempted to improve the therapeutic activity of the molecule. Of all such attempts to alter erythropoietin, none have succeeded in producing a molecule with enhanced *in vivo* biological activity or other improved properties for therapeutic applications.

The failure to identify a naturally-occurring alternate route of late stage erythropoiesis and the heretofore unsuccessful attempts to produce an erythropoietin analog with enhanced *in vivo* activity have provided little insight into how an improved erythrotropic molecule could be made.

Brief Description Of The Drawings

The present invention is described in connection with the attached drawings, in which:

Figure 1 is a schematic representation of the plasmid pEPOw5, the construction of which is described in Example 1;

Figure 2 is a schematic representation of the process, described in Example 1 and used to make, from plasmid pEPOw5(corrected) and plasmid SV2dhfrSVdeltaSJneo, the expression vector SV2dhfrSVdeltaSJneoEPO, which can be used to transform mammalian cells in culture to make native human erythropoietin;

Figure 3 is a graph illustrating the activities of native, "non-recombinant" human erythropoietin (hEPO Standard), native, "recombinant" human erythropoietin produced in culture by dhfr- Chinese hamster ovary cells which have been transformed with expression vector SV2dhfrSVdeltaSJneoEPO (rEPO), and the "recombinant" human erythropoietin analog pm25 with proline at residue 33 and cysteine at residue 139 (pm25), produced in culture by dhfr- Chinese hamster ovary cells which have been transformed with an analog of expression vector SV2dhfrSVdeltaSJneoEPO which includes DNA encoding the prepro form of such analog rather than the prepro form of native human erythropoietin; and

Figure 4 is a schematic illustration of both native human erythropoietin and pm25.

Detailed Description Of The Invention

In one of its aspects, the present invention is an analog of human erythropoietin which has the arginine residue at position 139 of the native glycohormone replaced with a cysteine residue.

In another of its aspects, the present invention is a further-modified analog wherein the cysteine residue at position 33 of wild-type erythropoietin is replaced with any one of the other 19 naturally-occurring amino acids, preferably proline.

In each instance, preferred examples of the analogs of the invention are those which lack the arginine residue at position 166 (*i.e.*, which are des-Arg^{166}).

In another of its aspects, the present invention is a double-stranded DNA sequence which comprises a segment of 498 or 495 nucleotides encoding an erythropoietin analog of the invention.

In a further aspect, the present invention entails a double-stranded DNA sequence comprising two, contiguous subsegments wherein a first subsegment is the above segment of 498 or 495 nucleotides and the other subsegment encodes the leader peptide of a mammalian preproerythropoietin, and wherein the two subsegments are joined such that, in the single polypeptide encoded by the contiguous subsegments, the carboxy-terminus of the leader peptide is adjacent the amino-terminus of the erythropoietin analog. Preferred leader peptides

sequence, where said second analog has amino acids that are different from those at the same positions in the sequence of native human erythropoietin, while said first analog is one having the same number and sequence of amino acids as said second analog except at either said first or said second position, where in the first analog the amino acid is the same as that found in the sequence of native human erythropoietin. The discovery of these "second analogs" of the invention rests on our discovery that a compensating change in amino acid sequence can be made which restores at least some activity lost due to the presence, in a "first analog", of an amino acid which is different from the amino acid found at the corresponding position in the native glycoprotein. It is anticipated that, in some instances, the second analog will be more active (*i.e.*, have greater *in vivo* specific activity in stimulating erythropoiesis) than the native glycohormone, while the first analog will be inactive *in vivo*.

The present invention also provides a process for making such a second analog of erythropoietin, described in the preceding paragraph, which process comprises (a) preparing a library of eukaryotic expression vectors, each of which comprises a cDNA sequence, positioned operably for expression in a mammalian cell, which (i) encodes a double mutant of native human preproerythropoietin (*i.e.*, a mutant with changes in amino acids at two positions from those present in the native glycohormone), (ii) comprises the triplet (codon) coding for the non-native amino acid of a first analog of native human erythropoietin, which first analog has no substantial activity in stimulating erythropoiesis, has the same number of amino acids as native human erythropoietin, but has at one position in its sequence an amino acid that differs from that found in the corresponding position in native human erythropoietin, and (iii) comprises a random mutation in a segment of cDNA which does not code for any part of the leader peptide of the native preproerythropoietin and does not include said triplet coding for said non-native amino acid of said first analog; (b) transfecting the library of expression vectors into mammalian cells for expression; and (c) selecting cells which secrete the desired second analog.

The present invention also provides a method for using such a second, double-mutant analog, with activity enhanced in comparison with a first, single-mutant analog of diminished activity relative to the native glycohormone, to make a third analog which has greater *in vivo* activity in stimulating erythropoiesis than the second analog. This process comprises changing the amino acid in said second analog, which is found in said first analog but not in native human erythropoietin, to the amino acid present at the corresponding position in native human erythropoietin.

The present invention further entails an analog of human erythropoietin which has the same number and sequence of amino acids as native human erythropoietin, except for a difference in amino acid at one position in said sequence, and which has greater activity in

sequences of the native human protein and the native human prepro-protein are given in SEQ ID N0:1.

The terms "native" and "wild-type", as used herein, are intended to be synonymous.

Standard abbreviations, as follows, are used herein for the 20 "naturally occurring" amino acids:

L-alanine	Ala
L-arginine	Arg
L-asparagine	Asn
L-aspartic acid	Asp
L-cysteine	Cys
L-glutamic acid	Glu
L-glutamine	Gln
glycine	Gly
L-histidine	His
L-isoleucine	Ile
L-leucine	Leu
L-lysine	Lys
L-methionine	Met
L-phenylalanine	Phe
L-proline	Pro
L-serine	Ser
L-threonine	Thr
L-tryptophan	Trp
L-tyrosine	Tyr
L-valine	Val

The standard, one-letter codes "A", "C", "G" and "T" are used herein to represent the nucleotides adenylate, cytidylate, guanylate and thymidylate, respectively. Those skilled in the art will understand that, in DNA sequences, the nucleotides are 2'-deoxyribonucleotide-5'-phosphates (or, at the 5'-end, triphosphates) while, in RNA sequences, the nucleotides are ribonucleotide-5'-phosphates (or, at the 5'-end, triphosphates) and uridylate (U) occurs in place of T. By "N" is meant any one of the four nucleotides.

A reference herein to an analog protein, "Protein X", as "[X^a, Y^b, des-Z^c]Protein X" means the analog in which the amino acid at position a in a native Protein X has been replaced with amino acid X, the amino acid at position b in the native Protein X has been replaced with amino acid Y, and the amino acid Z, normally present at position c in native Protein X, is missing.

As used herein, "SV2dhfrSVdeltaSJneo([X',Yb]hEPO)" means the expression vector SV2dhfrSVdeltaSJneoEPO with the cDNA coding for preproerythropoietin (see SEQ ID NO:1) replaced with a cDNA such that cultured mammalian cells transfected with the vector will secrete the [X^a, Y^b] analog of mature erythropoietin.

fragment was designed to have an HindIII site on the 5'-end and a unique EcoRI site on the 3'-end for ease of subsequent subcloning. After the ligation to provide the 640 bp fragment, that fragment was digested with EcoRI and partially digested with HindIII, and the resulting 640 bp fragment was ligated into similarly digested pUC19 to give plasmid pEPOw5, which is illustrated in Figure 1. The sequence of the 640 bp fragment in pEPOw5 was determined to attempt to verify that the preproerythropoietinencoding fragment would in fact encode human preproerythropoietin.

B. Correction of Synthesis Mistakes by Oligonucleotide-directed Mutagenesis.

The preproerythropoietin-encoding fragment of pEPOw5 contained two nucleotide errors, which resulted in amino acids changes at residues eighty-four and ninety-five from the amino acids present at those positions in human erythropoietin. To correct the errors, so that the amino acids at positions 84 and 95 would be the same as in human erythropoietin, required changing a C present at position 352 of SEQ ID N0:1 to a T; changing a T present at position 353 to a C; changing a C present at position 385 to a G; and changing an Apresent at position 387 to a G. Thus, pEPOw5 was digested with EcoRI to completion and with HindIII to partial completion. The digested plasmid was electrophoresed in a 0.7% agarose gel and a fragment of about 640 bp was electroeluted from the agarose into a 7.5M ammonium acetate salt bridge using a model UEA electroeluter (International Biotechnologies Inc., New Haven, Connecticut, USA) for one hour at 100 volts. The replicative form of M13mpl8 was digested to completion with HindIII and EcoRI and ligated to the eluted fragment. The ligated DNAs were transfected into *E. coli* (strain DH5alpha F') and the phage plaques were transferred to 2x YT media. Phage were propagated preparatively in *E. coli* DH5alpha F' cells. Phage were titred on *E. coli* CJ236 cells [dut-1, ung-1] and uracil containing phage prepared from the same strain by infection at a M.O.I. of0.2 as recommended by the manufacturer of the MutaGene mutagenesis kit (Bio-Rad Laboratories, Richmond, California, USA). Template DNA was extracted from the phage as recommended by the manufacturer. Mutagenesis of residues eighty-four and ninety-five was specified by simultaneous annealing of phosphorylated oligonucleotide-1, with the sequence of SEQ ID NO:2, and oligonucleotide-2, with the sequence of SEQ ID NO:3, to template DNA. DNA with the appropriate sequence corrections was synthesized *in vitro* as recommended by the manufacturer of the mutagenesis kit. The mutated (corrected) DNA was transfected into DH5alphaF' cells and phage plaques were isolated for DNA sequencing. After sequence confirmation, the mutated (sequence-corrected) preproerythropoietin-encoding DNA fragment was subcloned for expression as described below. The DNA sequence of the synthetic human preproerythropoietin-encoding DNA is shown in SEQ ID NO:1.

hour at 100 volts. The eluted DNA was ethanol-precipitated and resuspended in TE. The 5'-protruding ends were made blunt by enzymatic repair as described above for the expression vector. The blunted expression vector and the fragments were ligated with T4 DNA ligase at 15°C for 16 hours. The ligated mixture was transformed into *E. coli* and the correct clone identified by standard methods. The clones were propagated at the one-liter stage and plasmid DNA was prepared by lysis with sodium dodecyl sulfate (SDS) and cesium chloride density gradient centrifugation as described above. The plasmids (expression vectors) were stored in TE at 4°C. The expression vector for the human preproerythropoietin was designated SV2dhfrSVdeltaSJneoEP0, and that for the [Pro^{33}, Cys^{139}] human preproerythropoietin was designated SV2dhfrSVdeltaSJneopm25. A schematic representation of the construction and subcloning of SV2dhfrSVdeltaSJneoEP0 is illustrated in Figure 2.

SV2dhfrSVdeltaSJneo was constructed by addition of a neomycin resistance gene expression cassette and the SVdeltaSJ expression cassette to the publicly-available plasmid, pSV2-dhfr (American Type Culture Collection, Rockville, Maryland, USA, Accession No. 37146; Berg *et al.*, *Mol. Cell. Biol.* 1:854-864 (1981)). Plasmid pSV2-dhfr has a 2.3 kilobase pair (kbp) PvuII-to-EcoRI fragment (designated "ori PBR amp" in Figure 2), which was derived from pBR322 and has the bacterial origin of replication ("ori") and the beta-lactamase gene (which provides ampicillin resistance) ("amp") from plasmid pBR322. Plasmid pSV2-dhfr also has a 1.9 kbp expression cassette, which has a 0.34 kbp PvuII-to-HindIII fragment of simian virus 40 (SV40) DNA with the T-antigen promoter (designated "SVE" in Figure 2), a 0.74 kbp HindIII-to-BglI fragment with a cDNA sequence encoding mouse dihydrofolate reductase (designated "dhfr" in Figure 2), and a 1.6 kbp BglII-to-EcoRI fragment of SV40 DNA including a 0.82 kbp BglII-to-BamHI fragment having the SV40 T-antigen mRNA splicing and polyadenylation signals (designated "sv40 - A" in Figure 2) and a 0.75 kbp BamHI-to-EcoRI fragment having no known function (designated "sv40 - B" in Figure 2). The neomycin resistance gene expression cassette (to provide neomycin resistance to cells transformed with the vector) was inserted at the PvuII site of plasmid pSV2-dhfr by routine subcloning methods (*e.g.*, Maniatis *et al.*, *supra*). The neomycin resistance gene expression cassette is a 1.8 kbp fragment containing a 0.25 kbp PvuII-to-SmaI fragment of Herpes simplex virus-1 ("HSV1") DNA with the thymidine kinase promoter, a 1.0 kbp BglII-to-SmaI fragment of transposon TnS encoding the enzyme providing neomycin resistance, and a 0.6 kbp SmaI-to-PvuII fragment of HSV1 DNA encoding the thymidine kinase mRNA polyadenylation site and signal; all of these fragments are readily available to skilled practitioners of the art. The SVdeltaSJ expression casette is a 2.5 kbp fragment with the 0.34 kbp PvuII-to-HindIII fragment of SV40 DNA with the T-antigen promoter ("SVE" in Figure 2), an XbaI site for insertion of an heterologous DNA to be expressed under control of

centrifugation. The cell pellet is lysed by resuspension in saline containing 1% Triton X-100 and centrifuged to remove nuclei, with the resulting supernate containing total mRNA. Alternatively, the cell pellet can be lysed with guanidine isothiocyanate to prepare total RNA.

mRNA is then isolated by annealing to Dynabeads Oligo dt 25 (deoxythymidine) oligonucleotide-bearing beads as recommended by the manufacturer (Dynal A/S, N-0212 Oslo, Norway). Total RNA is prepared from guanidine isothiocyanate-lysed cells by standard methods. Complimentary DNA is synthesized from Oligo dt-selected mRNA isolated from approximately 10^5 cells or from approximately 0.1μg of total RNA using random hexamer primers and reverse transcriptase. The cDNA serves as template for a mutagenic polymerase chain reaction. The PCR reaction is carried out using three sets of primers, and the prepro-pm25 gene is synthesized as three distinct fragments (amino, middle and carboxy) with one set of primers for each fragment.

The 5' primer of the amino fragment contains restriction sites for subsequent molecular manipulations and sequences complimentary to the 5' end of the cDNA template. The 3' primer of the amino fragment contains a restriction site for molecular manipulation and sequences complimentary to the cDNA template.

The 5' primer of the middle fragment contains a restriction site capable of annealing the 3' restriction site of the amino fragment, a codon encoding the proline residue at position 33 of prepro-pm25, and sequences complimentary to the cDNA template. The 3' primer of the middle fragment contains a codon encoding a cysteine residue at position 139 of prepro-pm25, a restriction site for subsequent molecular manipulation, and sequences complimentary to the cDNA template.

The 5' primer of the carboxy fragment contains a restriction site capable of annealing to the 3' restriction site of the 3' primer of the middle fragment and sequences complimentary to the cDNA template. The 3' primer of the carboxy fragment contains sequences complimentary to the cDNA template and a restriction site for subsequent manipulations.

Each individual fragment can be subcloned and sequence confirmed prior to final assembly of the entire prepro-pm25 gene. The prepro-pm25 gene is assembled by restriction endonuclease digestion of the fragments using the restriction sites incorporated into each of the primers and ligation of the resulting complimentary ends. The assembled prepro-pm25 gene contains 5' untranslated and 3' untranslated sequences originally found in the message encoding erythropoietin as well as coding sequences of the prepro-pm25 gene. These untranslated sequences can be removed using subsequent PCR reactions with primers complimentary to the coding sequences of the prepro-pm25 gene, using methods known to those skilled in the art. The entire gene encoding prepro-pm25 is then subcloned into a suitable expression vector as previously described.

cells were sufficiently expanded to allow passage and continuous maintenance in F-12 minus medium G.

Amplification of the transfected erythropoietin or pm25 genes was achieved by stepwise selection of DHFR+, G418+ cells with methotrexate (reviewed by R. Schimke, *Cell* 37:705-713 (1984)). Cells were incubated with F-12 minus medium G, containing 150 nM methotrexate (MTX), for approximately two weeks until resistant colonies appeared. The MTX resistant cells were passaged and maintained in the appropriate selection medium. Further amplification was achieved by selection with 5 μM MTX, and cells were continuously maintained in the appropriate selection medium.

C. Maintenance and Storage of Cell Lines

Cells in culture and undergoing various selection or amplification procedures were re-fed with the appropriate culture medium three times weekly. Cells were passaged 1:5, with appropriate medium, into 75 cm^2 flasks using standard methods. Cryostorage was by resuspension of 2-4 x 10^6 cells in 1.8 ml of the appropriate culture medium containing 5% DMSO (Sigma, St. Louis, Missouri, USA) and cold storage for 24 hours at -80°C and then permanent storage at -135°C.

D. Production of Erythropoietin and pm25 in Serum Free Medium

Cells transfected with either the erythropoietin- (*i.e.*, rEPO-) or the pm25-expressing DNA were grown to confluence in F-12 minus medium G containing 300 micrograms/ml G418, then the culture media was removed and replaced with production medium (5 ml/25cm^2 of surface area). Production medium was VAS medium (serum-free culture medium supplemented with fish protamine sulfate) with L-glutamine, HEPES buffer, and without phenol red (JRH Biosciences). Cells were cultured at 37°C for three days and the conditioned medium was used as a source of rEPO or pm25.

Both the rEPO and the pm25 polypeptides obtained from the conditioned medium were des-Arg^{166}.

Example 5

In Vitro Biological Activity of Expressed Proteins

A. *In Vitro* Bioassay

Erythropoietin activity was determined by radiolabelled thymidine incorporation into spleen cells of phenylhydrazine-treated mice (G. Krystal, *Exp. Hematol.* 11:649-660 (1983)). Female C57/6 mice, at least ten weeks old, were injected intraperitoneally (ip) twice with

B. Radioimmunoassay and *In Vitro* Specific Activity

The masses of rEPO and pm25 were determined using a commercial radioimmunoassay kit (Incstar, Stillwater, Minnesota, USA) as described by the manufacturer, with the exception of inclusion of hEPO as a positive control for generating a standard curve. rEPO was purified to homogeneity as described below and the mass was determined by amino acid composition analysis. Standard hydrolysis was done using approximately 50-300 picomoles of protein under vacuum for 2 hours at 155°C using a Pico Tag Work station (Waters, Milford, Massachusetts, USA). The purified standard was stored at -80°C and a fresh aliquot used for the standard curve of each radioimmunoassay. The purified standard generated a linear response (log concentration vs. counts) when used at concentrations ranging from 0.25-2.0 ng/ml. At 2 ng/ml of standard, approximately 1050 counts were observed; at 1 ng/ml, approximately 2000 counts were observed; at 0.5 ng/ml, approximately 3200 counts were observed; and at 0.25 ng/ml, approximately 4250 counts were observed. Thus, the *in vitro* specific activity of both the rEPO or pm25 from culture supernates of transfected CHO cells was routinely calculated and ranged from 90,000 to 130,000 units/mg.

Example 6
In Vivo Biological Activity of Expressed Proteins

A. Wheat Germ Agglutinin Chromatography

Conditioned production media from cells transfected with each of rEPO, pm25 and HbSAg (as a negative control) were passed through a wheat germ agglutinin-Sepharose column to partially purify (approximately tenfold) and concentrate the erythropoietic activity. Thirty milliliters of conditioned medium was passed through a disposable minicolumn (Spectrum Medical Industries, Inc., Houston, Texas, USA), containing one milliliter of wheat germ agglutinin-Sepharose (Sigma) previously equilibrated with phosphate-buffered saline (PBS). The flowthrough was collected and passed through the column a second time, then the column was washed with nine column volumes of PBS and erythropoietic activity eluted with 1.5 column volumes of N,N-diacetylchitibiose (J.L. Spivak *et al.*, *Blood* 52:1178-1188 (1978). The eluted material was stored at -80°C until use in an *in vivo* bio-assay. The *in vitro* activity of the rEPO or pm25 from the wheat germ agglutinin chromatography was indistinguishable from the values obtained from conditioned production medium.

Table 2
In Vivo Bio-Assay Results

Sample	Treatment	% Reticulocytes	Treated/Control
Mock	T, W, Th	36	---
EPO	1.75u T, W, Th	75	2.08
EPO	5.25u T, W, Th	89	2.45
EPO	8.75u T, W, Th	116	3.20
pm25	1.75u T, W, Th	85	2.35
pm25	5.25u T, W, Th	119	3.29
pm25	8.75u T, W, Th	133	3.66

The increased potency of pm25 was also evident in animals treated with a single dose of pm25. Rats treated with a single dose of pm25 administered on a Tuesday showed a response substantially equivalent to that of rats treated with three doses of rEPO (*see* Table 3).

Table 3
In Vivo Bio-Assay Results

Sample	Treatment	% Reticulocytes	Treated/Control
Mock	T, W, Th	54	---
EPO	20ng T, W, Th	78	1.45
EPO	60ng T, W, Th	80	1.48
EPO	100ng T, W, Th	106	1.97
pm25	100ng T only	92	1.71

Rats treated with a single dose of rEPO on Tuesday, Wednesday or Thursday, when compared to animals treated with three doses of rEPO (one on each day), showed no such equivalence (*see* Table 4).

Standardization compensated for the day-to-day variation in instrument settings. A computer protocol was established to collect three histograms; dual-parameter log side scatter vs. log forward scatter, log fluorescence vs. log forward scatter, and single-parameter log green fluorescence.

A stained sample was analyzed to establish gates for inclusion of only erythroid cells. An amorphous gate was drawn around a population containing lymphoid and erythroid cells on the log forward scatter vs. log side scatter plot, which eliminated platelets and background debris. This gated population was then represented on a log fluorescence vs. log forward scatter histogram. A rectangular gate was drawn around the negative erythroid population and the positively staining reticulocyte population, but excluding the highly staining lymphoid population. The gated erythroid population was represented on a single-parameter log green fluorescence histogram. An unstained control sample was analyzed on the instrument and 25,000 events were collected. The cursor was placed to include 0.1% of the autofluorescing cells and the stained samples were analyzed. Reticulocytes were expressed as a percentage of all erythroid cells.

Example 7
Purification of Expressed Proteins

A. Purification of rEPO

Proteing rEPO was purified from conditioned production media by a combination of ion-exchange, wheat germ lectin, and reverse phase chromatography. Typically, ten liters of conditioned medium were clarified by centrifugation and then concentrated ten-fold using a Benchmark rotary concentrator (Membrex, Garfield, New Jersey, USA) with a 10,000-dalton molecular weight cut-off membrane at 4°C. The concentrated harvest was centrifuged at 15,000 x g for thirty minutes, then diluted with an equal volume of cold distilled water containing 25 KIU (kilo international units) of aprotinin per milliliter, after which the pH was adjusted to 7.3-7.4, if necessary. The diluted concentrate was passed over two ion-exchange columns connected in series. The first column was an S-Sepharose Fast Flow resin (Pharmacia-LKB, Inc., Piscattaway, New Jersey, USA) and the second a DEAE Sepharose Fast Flow resin (Pharmacia-LKB). The columns were each 1.6 x 33 cm and were equilibrated with 20 mM NaH_2PO_4, pH 7.4, 20 mM NaCl. Under these conditions, rEPO did not bind to either column; however, a substantial purification was achieved since many other proteins did bind. The flow-through and a 200 ml wash were loaded onto a 20 ml wheat germ agglutinin-Sepharose column (Sigma) previously equilibrated with 20 mM NaH_2PO_4, pH 7.4, 20 mM NaCl and washed exhaustively with buffer containing 135 mM NaCl. rEPO was eluted with

urea. The sample was loaded onto a Mono-S cation exchange column (Pharmacia) (1.6 x 50 mm), equilibrated in the same buffer, and chromatographed at room temperature using a SMART system. The sample was loaded at 100 microliters per minute and chromatographed at 150 microliters per minute. Five minutes after loading, a twenty minute gradient from 0-50% eluting buffer (100 mM $CH_3C0_2NH_4$, pH4.1, 1M NaCl, 6M urea) was performed. Fractions were collected using the SMART system "peak detection" capability and SDS-PAGE was used to identify pm25. Eluted pm25 was then re-chromatographed on a URPC C2/C18 column (2.1 x 100mm) as described above. The sample was acidified with 5% TFA and loaded onto the column at 200 microliters per minute. Initial buffer was 0.1% TFA in 5% CH_3CN and the elution buffer was 0.08% TFA in 80% CH_3CN. Five minutes after loading, a 25 minute gradient elution (from 0 to 100% buffer) was performed. The SMART system "peak detection" capability was used to collect fractions and SDS-PAGE performed to locate and assess purity. Electrophoretic analysis revealed a single band migrating between the 29,000 and 43,000 dalton markers with an apparent molecular weight of approximately 36,000 daltons. Subsequent protease digestion with Lys-C and sequence analysis revealed the presence of pm25 and no other proteins.

Example 8
Biological Activity of Purified Proteins

A. *In Vivo* Activity of rEPO and pm25 in a Non-Anemic Rat Model

The biological activities of purified rEPO and pm25 were compared in a long-term, non-anemic rat model by measuring hematocrits of treated and mock-treated rats. Groups of five rats were treated three times per week with rEPO or pm25 or were mock-treated with vehicle (PBS containing 0.2% BSA) for a total of four weeks. Four groups of animals were treated by intravenous injection with 150, 300, 450 or 600 ngs of rEPO in vehicle and two groups treated in the same way with 150 or 300 ngs of pm25 in vehicle. One group of animals served as controls and were treated intravenously with vehicle three times per week for four weeks. Hematocrits were determined for each animal at the end of the four week period and the average hematocrit values were calculated for each group. The animals treated with 150 ngs of pm25 showed a response substantially equivalent to that of animals treated with 300 ngs of rEPO. Similarly, animals treated with 300 ngs of pm25 showed a response substantially equivalent to that of animals dosed with 600 ngs of rEPO. In this long-term model, therefore, pm25 was approximately twice as effective in raising the hematocrit of treated animals as native recombinant erythropoietin (*see* Table 5).

In yet another related experiment, single weekly dosing of rEPO was tested by administering rEPO once per week in a long-term, non-anemic rat model and comparing hematocrits of mock-treated and rEPO-treated animals. Groups of five animals were mock-treated with vehicle (PBS containing 0.2% BSA), treated with rEPO three times per week, or treated with rEPO once weekly for a total of four weeks.

One group of animals was treated with vehicle alone three times per week for four weeks and served as the vehicle control. Three groups of animals were treated with 150, 300 or 450 ngs of rEPO in vehicle three times per week for four weeks. Three additional groups of animals were treated with 300, 600 or 900 ngs of rEPO in vehicle once weekly for four weeks.

The animals treated three times weekly showed a dose-dependent increase in hematocrit levels ranging from 55.3% to 60.8% (*see* Table 7). Animals treated with rEPO at 300 or 600 ngs once per week showed no appreciable increase in hematocrit compared to the vehicle treated controls; however, animals treated with 900 ngs once weekly showed only a modest increase in hematocrit as compared to vehicle-treated animals and a significantly lower increase than that found in animals treated with the lowest dose of rEPO administered three times per week. These data, when compared to those obtained with once-weekly administration of pm25 as shown in Table 6, suggest that the analogs of the present invention produce greater *in vivo* erythropoietic effects upon once-weekly dosing than native erythropoietin.

Table 7

Long-term *In Vivo* Bio-Assay Results

Sample	Treatment	Hematocrit (Final)
Mock	3 times weekly (TTW)	51.0
EPO	150ng TTW	55.3
EPO	300ng TTW	57.0
EPO	450ng TTW	60.8
EPO	300ng once weekly (QW)	51.3
EPO	600ng QW	50.5
EPO	900ng QW	53.0

Example 9

Structural Characterization of Expressed Proteins

A. Protease Lys-C Digestion of Purified rEPO and pm25

rEPO and pm25 were digested with Lys-C (K.L. Stone *et al.*, A Practical Guide to Protein and Peptide Purification for Microsequencing, pp. 31-471, ed. P.L. Maztsudaira, Academic Press (1989)), and the resulting peptides were analyzed to map the location of disulfide bonds in these molecules. Typically, 100 micrograms of purified protein was dried into a microcentrifuge tube and the protein dissolved with 50 microliters of 400 mM NH_4HCO_3, pH 8.2, 2 mM EDTA, 8M urea (deionized). A second sample of each was reduced prior to Lys-C digestion. Reduction was done with dithiothreitol (DTT) at 4.5 mM for 30 minutes at 37°C under nitrogen; after reduction, the sample was equilibrated to room temperature and alkylated with 10 mM iodoacetic acid for 1 hour at room temperature under nitrogen and in the dark. The reduced and non-reduced samples were diluted with distilled water to bring the urea concentration to 2M. The proteins were digested with 1.5 micrograms of Lys-C for 2-3 hours at 37°C under nitrogen, then an additional 1.5 micrograms of enzyme was added and the digestion was continued for 15 hours. The digestion was terminated by addition of TFA to 0.5%. Peptides were isolated by reverse phase high performance liquid chromatography (HPLC) using a uRPC C2/C18 column (2.1 x 100 mm) and a SMART system (Pharmacia). The equilibration buffer was 0.1% TFA in 5% CH_3CN and the elution buffer was 0.08% TFA in 80% CH_3CN. The flowrate was 200 microliters per minute. Five minutes after loading, a 55 minute gradient elution (from 0 to 100% buffer) was performed. Eluted peaks were collected with the SMART system "peak detection" capability. Fractions were stored at -20°C prior to amino acid sequencing. The identity of the Lys-C peptides was determined by amino acid sequencing (R.M. Hewick *et al.*, *J. Biol. Chem.* 256:7990-7997 (1981), using an ABI Model 470A or 477A sequenator equipped with an ABI Model 120A PTH analyzer (Applied Biosystems, Inc., Foster City, California, USA). Data were collected and analyzed using a PE Nelson software system for amino acid sequence analysis (Access*Chrom, Micro Vax 2000, Cupertino, California, USA) with a software package for chromatographic data management.

B. Identification of Disulfide Bond Positions in rEP0 and pm25

The deduced amino acid sequence of erythropoietin predicts eight lysine residues in the molecule. The construction of pm25, described in Example 1, does not alter the number or location of the lysine residues. Therefore, both of these molecules should have very similar Lys-C peptides, with any differences arising because of the amino changes at residues 33 and

compensating mutations are possible in mammalian erythropoietins, makes available a wide variety of double mutants of that glycohormone, including such double mutants as are substantially improved in erythropoietic activity over that of the corresponding, wild-type glycohormone.

Beginning with a first cDNA encoding a first mutant, which has reduced activity (and typically substantially no activity) because of a change in an amino acid at one position, the skilled practitioner can readily generate very large numbers of second mutants, which differ from the first in having one or more changes in amino acids at second or subsequent positions, and can then expression-screen the second cDNAs encoding the second mutants for those mutants which have the desired level of erythrotropic activity.

The process will be illustrated with human erythropoietin and for the typical case, in which the first mutant has no erythropoietic activity *in vitro*. However, it will be readily apparent that the process can be applied with any mammalian erythropoietin and for cases in which the first mutant has activity that is reduced in comparison with the wild-type but not eliminated.

The process entails four steps, beginning with a first cDNA, which encodes a prepro-first mutant of human erythropoietin. Typically, the segment of the cDNA encoding the leader peptide will encode the leader peptide for prepro-human erythropoietin. In a first step, a large number of random mutations in the first cDNA are generated at sites such that any resulting change in amino acid will be at a position, in the primary sequence of the mature glycoprotein, which is distant from the position of the change in amino acid in the first mutant. Even when the mutations are essentially randomly distributed along the entire cDNA encoding the prepro-first mutant, most of them will be in nucleotide triplets (codons) that are outside the leader peptide-encoding segment of the first cDNA and that correspond to amino acids at positions which are distant, in the primary sequence of the mature protein, from the position of the amino acid change in the first, inactive mutant glycohormone. By "distant" is meant a separation of at least 1 and more typically at least 10 amino acid positions.

Second, the repertoire of randomly-mutated second cDNAs from the first step is ligated for cloning into an eukaryotic expression vector and the resulting library ("random library") of vectors harboring the randomly-mutated second cDNAs is cloned in a suitable host to prepare a convenient quantity of vectors of the library.

Third, the random library is transfected into eukaryotic cells (e.g., CHO cells or other suitable mammalian cells), in which the randomly-mutated second cDNAs in the expression vectors of the libraty are capable of being expressed and processed to secrete mature, second mutants. The cells are then cultured, and the resulting cell population is screened to isolate single-cell clones which produce erythropoietin activity in an *in vitro* assay for such activity.

More specifically, the process is carried out with reference to the double mutant pm25 as follows: A first cDNA encoding an inactive or reduced-activity erythropoietin analog, such as one having a Cys-to-Pro mutation at position 33, is used as a template for starting the mutagenesis using PCR. One such first cDNA has the sequence which differs from that shown in SEQ ID NO:1 only by having CC at positions 199 and 200. Using this first cDNA for illustration, a first PCR primer is used which hybridizes to a segment, including the CC at positions 199 and 200, of the strand of the cDNA which has (except at positions 199 - 200) the sequence shown in SEQ ID NO:1. Also, a second primer is used which hybridizes to that strand of the cDNA to which the first primer does not anneal. The second primer anneals to a segment of this strand which has its 3'-end base-paired to a base pair that is at a position located 3' of base 517 as illustrated in SEQ ID NO:1. Thus, in the PCR mutagenesis process, it is possible that the base pair of the first cDNA corresponding to base 517 in SEQ ID NO:1 can be mutagenized to a T, which will convert the triplet 517-519 to one coding for Cys.

The PCR-mutagenized products, which include some sequences with random mutations between the primers defining the ends of the amplified product, are then digested with restriction enzymes using sites incorporated into the PCR product as suggested above. The fragments from the digestion which are of a size that includes those with the random mutations are then ligated into a suitable eukaryotic expression vector, operably for expression and secretion from a mammalian cell in culture of the doubly mutated erythropoietin analogs. Such an expression vector will provide a mammalian (in this case, preferably human) erythropoietin leader peptide at the amino-terminus of the mature, double mutants which provides for their glycosylation and secretion and, of course, will provide appropriate signals for transcription and other steps necessary for expression of the preproerythropoietin double mutants in a mammalian cell. The vector will also be suitable for cloning to provide sufficient qualities of the vector for mammalian cell transfection and other uses. For example, the PCR-amplified fragments can first be ligated to fragments which code for parts of preproerythropoietin, providing fragments with cDNA coding for full-length preproerythropoietin double mutants. These fragments, coding for full-length protein, can then be ligated into a vector such as SV2dhfrSVdeltaSJneo to provide suitable expression vectors for the double mutants.

The resulting library of expression vectors, with the doubly mutated cDNAs incorporated, is then cloned in a suitable host, such as *E. coli*, to obtain sufficient amounts of the library for further work.

The library of expression vectors, with the doubly mutated cDNAs, is transfected into mammalian cells (e.g., CHO cells) as described above in Example 2. The cells are cultured as single-cell clones or colonies of small numbers (e.g., about 10) of cells. Each culture is then

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Okasinski, Gregory F.
DeVries, Peter J.
Mellovitz, Barry S.
Meuth, Joseph L.
Schaefer, Verlyn G.

(ii) TITLE OF INVENTION: Erythropoietin Analog Compositions and Methods

(iii) NUMBER OF SEQUENCES: 15

(iv) CORRESPONDENCE ADDRESS:
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(B) STREET: Dept. 377 - AP6D One Abbott Park Road
(C) CITY: North Chicago
(D) STATE: Illinois
(E) COUNTRY: United States
(F) ZIP: 60064-3500

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: MS-DOS Ver. 5.0 ASCII Text Editor

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/055,076
(B) FILING DATE: 29-APR-1993
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Weinstock, Steven W.
(B) REGISTRATION NUMBER: 30,117
(C) REFERENCE/DOCKET NUMBER: 5282.US.01

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (708) 937-2341
(B) TELEFAX: (708) 938-2623

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 625 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

CGC GGA AAG CTG AAG CTT TAC ACA GGG GAG GCA TGC AGG ACA 591
Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala Cys Arg Thr
150 155 160

GGG GAC AGA TGATGACCAG GTGTTACCTG GATCC 625
Gly Asp Arg
165

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCCTGTTGGT CAATTCCTCC CAGCCGTG 28

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCTGCAGCTG CATGTGGATA AAGCCGTCAG 30

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCTGTGCTGA GCACCCCAGC TTGAATGAGA AT 32

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 85 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGCTTGTGTG GATCCCCGCC AGGCGCCACC ATGGGGGTGC ACGAATGTCC 50

TGCCTGGCTG TGGCTTCTCC TGTCCCTGCT GTCGC 85

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 82 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TCGCTCCCTC TGGGCCTCCC AGTACTGGGC GCCCCACCAC GCCTCATATG 50

TGACTCGCGA GTCCTCGAGA GGTACCTCTT GG 82

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 85 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTGGAGGCCA AGGAGGCCGA GAATATTACG ACGGGCTGTG CTGAGCACTG 50

CAGCTTGAAT GAGAATATCA CTGTCCCAGA CACCA 85

(ii) MOLECULE TYPE: syntheitc DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GATGCGGCCT CAGCTGCTCC ACTCCGAACA ATCACTGCTG ACACTTTCCG 50

CAAACTCTTC CGAGTCTACT CCAATTTCCT CC 82

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 85 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CTCCGCGGAA AGCTGAAGCT TTACACAGGG GAGGCATGCA GGACAGGGGA 50

CAGATGATGA CCAGGTGTTA CCTGGATCCT GAATT 85

10. A double-stranded DNA according to any one of Claims 5-9 wherein the segment of 498 or 495 nucleotides is one of two, contiguous subsegments and the other subsegment encodes a leader peptide of a mammalian preproerythropoietin and is joined to the 498-nucleotide or 495-nucleotide subsegment such that, in a polypeptide encoded by the contiguous subsegments, the carboxy-terminus of the leader peptide is adjacent the amino-terminus of the erthyropoietin analog.

11. A double-stranded DNA according to Claim 10 wherein the leader peptide encoded by the leader-peptideencoding subsegment has the sequence Met Gly Val His Glu Cys Pro Ala Trp Leu Trp Leu Leu Leu Ser Leu X^3 Ser Leu Pro Leu Gly Leu Pro Val X^4 Gly, wherein X^3 is selected from the group consisting of Leu and Val, and X^4 is selected from the group consisting of Leu and Pro.

12. A double-stranded DNA according to Claim 11 wherein X^3 and X^4 are each Leu.

13. A double-stranded DNA according to Claim 10 which is an expression vector for expressing, in a mammalian cell in culture, a preproerythropoietin analog having a sequence consisting of the sequence of the leader peptide joined at its carboxy terminus to the amino terminus of the sequence of the analog of human erythropoietin.

14. A double-stranded DNA according to Claim 11 which is an expression vector for expressing, in a mammalian cell in culture, a preproerythropoietin analog having a sequence consisting of the sequence of the leader peptide joined at its carboxy terminus to the amino terminus of the sequence of the analog of human erythropoietin.

15. A double-stranded DNA according to Claim 12 which is an expression vector for expressing, in a mammalian cell in culture, a preproerythropoietin analog having a sequence consisting of the sequence of the leader peptide joined at its carboxy terminus to the amino terminus of the sequence of the analog of human erythropoietin.

16. An expression vector according to Claim 15 which consists of SV2dhfrSVdeltaSJneo with, inserted at the Xbal site of that vector operably for expression of a preproerythropoietin analog, a double-stranded DNA which comprises a segment which encodes the preproerythropoietin analog.

25. A pharmaceutical composition useful for treating anemia, comprising (a) a therapeutically effective amount of an analog of human erythropoietin which has an amino acid sequence selected from the group consisting of the sequences of [X^{33}, Cys^{139}, des-Arg^{166}]-human erythropoietin, wherein X^{33} is selected from the group consisting of the 20 naturally-occurring amino acids, in combination with (b) a pharmaceutically acceptable carrier.

26. A pharmaceutical composition according to Claim 25 wherein X^{33} is Pro.

27. A pharmaceutical composition according to Claim 25 wherein X^{33} is Cys.

28. A second analog of human erythropoietin, which has specific activity in erythropoiesis that is significantly greater than that of a first analog, which first analog has specific activity in erythropoiesis that is significantly less than that of native, human erythropoietin, said second analog having the same number and sequence of amino acids as native human erythropoietin except at a first and a second position in said sequence, where said second analog has amino acids that are different from those at the same positions in the sequence of native human erythropoietin, and said first analog having the same number and sequence of amino acids as said second analog except at either said first or said second position, where the amino acid is the same as that in the sequence of native human erythropoietin.

29. A second analog according to Claim 28 which has erythropoietic activity that is substantially the same as or greater than that of native human erythropoietin and for which the first analog has no or substantially-reduced erythropoietic activity.

30. A process of making a second analog of erythropoietin according to Claim 29, which process comprises the steps of:

(a) preparing a library of eukaryotic expression vectors, each of which comprises operably for expression in a mammalian cell a cDNA which (i) encodes a double mutant of native human preproerythropoietin, (ii) comprises the triplet coding for the amino acid of a first analog of native human erythropoietin, which first analog has no or reduced erythropoietic activity and has the same number of amino acids as native human erythropoietin but for one position in its sequence at which an amino acid differs from that at the corresponding position in native human erythropoietin, and (iii) comprises a random mutation in a segment which does not code for any part of the leader peptide of the native preproerythropoietin and does not include said triplet coding for said amino acid of said first analog;

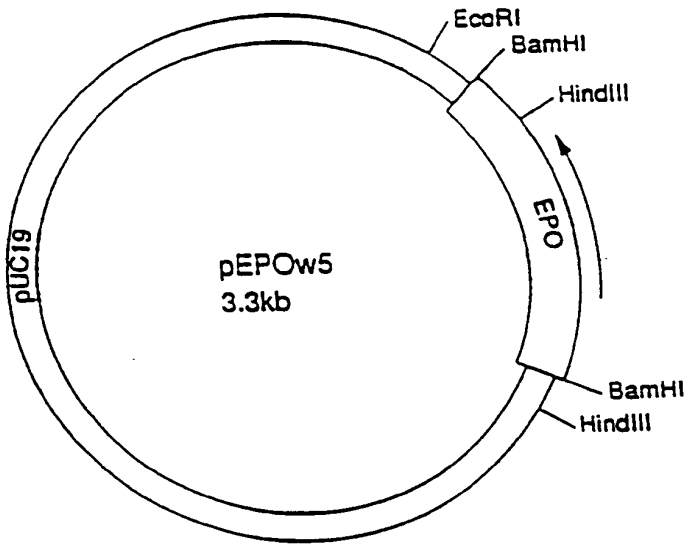

Figure 1

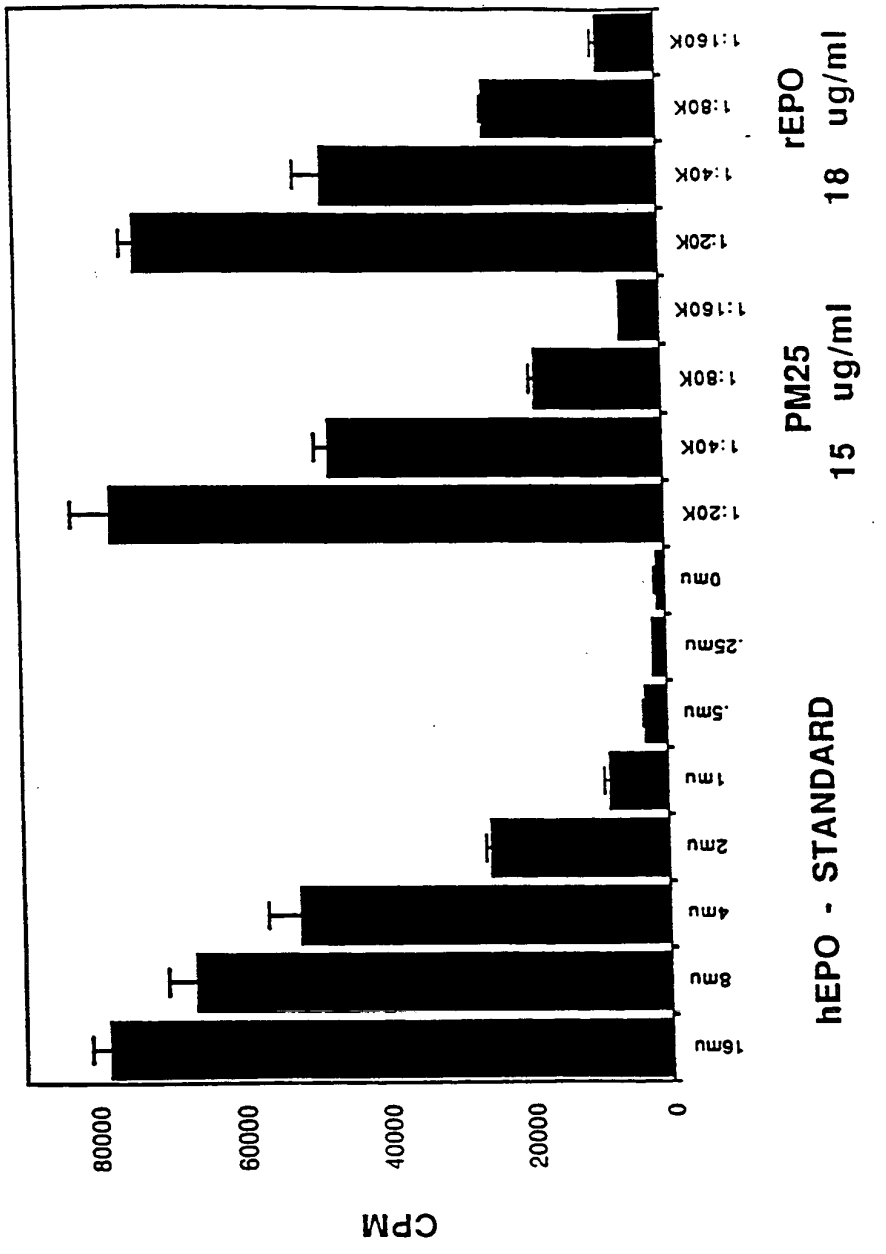

Figure 3

INTERNATIONAL SEARCH REPORT

International application No
I US94/04755

A CLASSIFICATION OF SUBJECT MATTER
IPC(5) :A61K 37/02; C07K 13/00; C12N 15/19, 15/63, 5/10
US CL :530/351, 397; 514/8; 424/85.1; 435/69.1, 69.5, 320.1, 240.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/351, 397; 514/8; 424/85.1; 435/69.1, 69.5, 320.1, 240.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

US PTO-APS; Dialog (Medline, Biosis, CAS, WPI, SciSearch)
Search terms: erythropoietin, EPO; mutein, mutat?, mutagen?, site directed, analog?; recombinant, cDNA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	*Molecular and Cellular Biology*, Volume 6, Number 3, issued March 1986, J. F. McDonald *et al.*, "Cloning, Sequencing, and Evolutionary Analysis of the Mouse Erythropoietin Gene", pages 842-848, especially Fig. 4, Fig. 6, and the paragraph bridging columns on page 847.	1-29, 32
Y	*Progress in Clinical and Biological Research*, Volume entitled "The Biology of Hematopoiesis", issued 1990, J.-P. Boissel *et al.*, "Erythropoietin structure-function relationships", pages 227-232, especially the first full paragraph on page 228.	1-29, 32
Y	*Biochimica et Biophysica Acta*, Volume 1171, issued 1992, M. Nagao *et al.*, "Nucleotide sequence of rat erythropoietin", pages 99-102, especially Fig. 2 and page 100, col. 1.	1-29, 32

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
03 AUGUST 1994	19 AUG 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DAVID L. FITZGERALD Telephone No. (703) 308-0196

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